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Ectyonin, an Antimicrobial Agent from the Sponge, *Microciona prolifera* Verrill

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(Plate I)

Introduction

It is generally recognized that external metabolites may play a regulatory role in aquatic ecology by either inhibiting or promoting growth (Nigrelli, 1958). Previous studies in the laboratory of Marine Biochemistry and Ecology have indicated that a number of marine invertebrates produce such substances. Holothurin, a toxic steroid saponin isolated by Nigrelli (1952) from the Bahamian sea cucumber, *Actinopyga agassizi* Selenka, is an example of such biologically-active material.

In work (unpublished) done in 1954 and 1955, the senior author found that simple water extracts of several species of Atlantic sponges were capable of inhibiting growth of certain marine Gram-negative bacteria and *Micrococcus aureus*. The degree of inhibition varied with the source of the active principle and other uncontrolled factors. The present report deals with a broad spectrum antimicrobial agent extracted from the red-beard sponge, *Microciona prolifera*, commonly found along the Atlantic coast of North America. Ectyonin, the name proposed for this substance, is derived from the name of the sub-family Ectyoninae.

Materials and Methods

Preparation of the Sponge.—The sponges were collected from July through November. Colonies, ranging from one to three feet in width and up to one foot in height (Fig. 1)[1], were quickly washed with sea water to remove superficial impurities; the inhabiting fauna (commensals and epibionts) was manually removed. The latter consisted of worms (*Clymenella, Lepidonotus, Spirorbis*), crustaceans (*Carcinides maenas, Panopeus, Balanus* and isopods), clumps of mussels (*Modiolus*), sea anemones (*Sagartia*), some bryozoans and gobioid fish. Some of the cleaned sponge was stored in a freezer wrapped in aluminum foil.

Extractions were made of fragments and homogenates of fresh and frozen sponges, and of oven-dried (over-night at 60-80° C.) material that was fragmented or pulverized. Sponges kept in running sea water for a few weeks were also extracted; the color of this material changed from bright brick red to purplish black, possibly indicating some decomposition.

Preparation and Purification of Extracts.—The following procedures were found most satisfactory under our laboratory conditions. Dried sponges were cut into small pieces or pulverized by mortar and pestle or Waring blendor. Extraction was made with ethyl ether for 24 hours at 7° C. The orange-colored extract was decanted and decolorized with Norit-A for approximately one hour at room temperature. The total solids were determined before and after decolorization, and the material was then concentrated by evaporation. Extracts concentrated without decolorizing were thick and viscid; such materials were difficult to decolorize.

Homogenates of fresh sponges were made with hot and cold distilled water, and fractionated by centrifugation at 10,000 to 15,000 rpm, or extracted with 95% ethanol, acetone, chloroform, benzene, petroleum ether and ethyl ether, and then centrifuged. Intractable emulsions were obtained in some cases. The water in ethyl ether extracts of living cell suspensions and fragmented and homogenized fresh sponges was

[1]These are considerably larger than those reported by Hartman, 1958.

removed with sodium sulfate. Large quantities of dried sponge were also extracted in Soxhlet with ethanol, chloroform and acetone, evaporated and re-extracted with a small volume of ethyl ether. Purification included removal of pigments with Norit-A, filtration and hydrolysis with cold N/100 NaOH.

The components of the ethyl extracts were chromatographed on paper with ethyl ether and petroleum ether used as ascending phases. Petroleum ether and acetone fractions of purified ether extracts were also collected from an alumina adsorption column (80-200 MM). Concentrated decolorized ether extracts of known antimicrobial activity were used to obtain crystals at room temperature and at 0° C.

Microbiological Testing Methods. — Filter paper discs 13 mm. in diameter were repeatedly impregnated with solutions to be tested, or smeared with semi-solid fractions, and then dried at room temperature. Control discs were impregnated with the solvents. The test organisms were *Micrococcus aureus, Escherichia coli, Pseudomonas pyocyanea, Klebsiella pneumoniae, Mycobacterium* from Cobra, *Mycobacterium* 607 (Bovine) and *Candida albicans.* Plates were poured with 1 ml. of a 24-hour culture in 20 ml. nutrient agar; discs were placed on the solidified medium and the plates incubated at 37° C. for 24-48 hours, and longer for *Mycobacterium* and *Candida.* Inhibition zones were recorded in mm., measuring from the edge of the discs. The chromatographic paper strip containing the components of the ethyl ether extract were also tested in this manner, as were discs impregnated with sesame oil suspensions of evaporated, decolorized, ethyl ether extracts. The latter material was also used for the *in vivo* tests.

In Vivo Tests for Toxicity and Antimicrobial Action.—The tests were limited to a few animals in view of the difficulties encountered in preparing sufficient amounts of ethyl ether extracts under our laboratory conditions. A suspension of the active material was made by evaporating 20 ml. of decolorized ether extract on 0.5 ml. of sesame oil. Paper discs dipped in this suspension were found to produce a 7 mm. zone of inhibition against *Pseudomonas pyocyanea.* Adult killifish, *Fundulus heteroclitus,* two inches long, were injected intraperitoneally with 0.02 ml. of the suspension; four fish were simultaneously injected with 0.02 ml. of a 24-hour-old broth culture of *Pseudomonas* and two fish were given the bacterial suspension only. Two hybrid AB mice were injected subdermally in the axillary region with 0.2 ml. of the suspension.

Observations

In vitro tests for antimicrobial activity were made of all extracts and their fractions. The active substance appears to be only slightly soluble in water, chloroform and acetone. It is best obtained with ethyl ether from living cell suspensions, fragments of fresh or frozen sponge, or from evaporated Soxhlet extracts of dried material. It has been found necessary to decolorize the extracts prior to reducing them in volume, even though the original pigment-containing ethyl ether extracts produced striking inhibition zones in all organisms tested (Fig. 2A). Discs impregnated with large amounts of the thick pigmented extracts failed to produce inhibition in proportion to the theoretically determined amounts of the inhibiting agent (Fig. 2D).

No quantitative data were obtained in the early phase of this work. The information in Table 1 was derived from analysis of sponges collected on specific dates. In each case extracts were made of oven-dried fresh and frozen sponges. Comparable weights of dry sponge were used; the original wet weight was approximately 180 g. Usually the dry weight represents ⅕th-⅛th of the wet weight of the sponge.

Table 1.

	Method of Storage			
	Frozen		Dry	
Date of collection	9/15/58	11/8/58	9/15/58	11/8/58
Dry weight (g.)	31.0	33.0	31.0	33.0
Total ether-extracted solids (mg.)	138.5	107.5	737.0	616.0
Total solids after decolorizing (mg.)	11.85	7.05	77.0	32.0

The *in vitro* activity was proportionate to the total solids per ml. of decolorized ethyl ether extract of sponge of the same collection (Table 2). Materials washed or handled too long before freezing and drying yielded extracts of low antimicrobial activity but the solids per ml. were higher. Although non-decolorized, water-free ethyl ether extracts of fresh sponge collected in July (7/21/58) produced zones of inhibition up to 27 mm., these results could not be repeated with sponge collected in the autumn. Decrease in the relative amounts of the antimicrobial substance was also obtained when the

sponge was allowed to start decomposing in running sea-water; extracts of healthy and such partly decomposed sponge produced inhibition zones of 10-14 mm. and 1.5-3 mm., respectively.

When the amount of ether-extractable decolorized solids present on a disc was estimated on the basis of total solids per ml. of extract, the results are shown in Fig. 3 and were as follows:

Table 2.

Date of Collection	Estimated Decolorized Solids/disc	Av. Inhibition
9/15/58	7.9 mg.	5.0 mm.
	3.9	2.0
11/8/58	4.7	4.5
	2.35	2.0

As shown in Table 1, sponges collected in September produce relatively more ether extractable solids than sponges obtained in November, although there seems to be considerable difference in the total solids obtained from frozen and dried material. This may be due to slight difference in the extraction method. From these and other data it appears that the solids in decolorized ether extracts represent from 0.04 to 0.10% of the dry weight of the sponge. Materials removable by cold NaOH treatment represents slightly over 90% of the solids in the decolorized ethyl ether extracts.

The decolorized ether extracts that showed antimicrobial activity produced a mixture of crystals when evaporated at room temperature; at 0° C. white crystals were formed on the strip of filter paper immersed in the container. No antimicrobial activity was demonstrated, but this may be due to the small amount of crystals collected in this manner.

Decolorized extracts treated with NaOH, or fractions collected on an alumina column, showed no antimicrobial activity. The extract distributed along the chromatographic paper strip produced a characteristic pattern of inhibition when applied to a seeded plate. The greatest zone of inhibition occurred on either side of the middle part of the strip and it tapered towards the starting and terminal points. The suspension of ethyl ether extract in sesame oil placed on the disc produced a 7 mm. zone of inhibition.

With the exception of one death due to injury, all fish injected with both bacteria and sesame oil suspension of the active extract survived. Fish injected with bacteria alone died in a relatively short time. One mouse developed a nodule at the site of injection after several weeks but otherwise showed no ill effects from the intradermal injection of the sesame oil suspension of the sponge extract. There was some indication that the fish infected with *Pseudomonas* may have been protected by Ectyonin. Further tests, however, are needed to evaluate the *in vivo* effects.

Discussion

Microciona prolifera is relatively abundant from early June through November along the coast of the western North Atlantic. Although no attempts were made during the early phase of this work to quantitate the active principle in this sponge, the data obtained from materials collected in September and November indicate that the yield decreases in the colder months. To what extent this may be related to the natural metabolic cycle of the sponge or to environmental conditions has not been determined.

The solids removable by cold NaOH treatment, and presumably representing over 90% of the total ethyl ether extractable solids after decolorizing, showed no antimicrobial properties in the *in vivo* tests. This may be due either to the extremely low yields after this procedure or to the possibility that the antimicrobial property is associated with the lipoid fraction. It should be mentioned here that *Microciona* contains a variety of sterols (Bergmann *et al.*, 1945).

The active principle in *Microciona prolifera* is remarkable for its activity against a variety of microorganisms, including Gram-positive, Gram-negative, and acid-fast forms, as well as *Candida albicans*. Of special interest is its activity against *Pseudomonas pyocyanea*. Under laboratory conditions, water extracts of *Microciona* were not significantly antimicrobial but it is suggested that under natural conditions Ectyonin may play some role in the biochemical ecology of the sponge.

Summary

Ectyonin, a fraction of the ethyl ether extracts of the red-beard sponge, *Microciona prolifera*, showed antimicrobial properties when tested *in vitro* on Gram-positive, Gram-negative, acid-fast bacteria, and *Candida albicans*. Preliminary tests indicate that it is not toxic to fish and mice.

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EXPLANATION OF THE PLATE

PLATE I

FIG. 1. Collection of large colonies of *Microciona prolifera* from the Long Island coast in late summer.

FIG. 2. **A.** Zone of inhibition of *Pseudomonas pyocyanea* produced by crude ethyl ether extract of unknown concentration from sponge collected in mid-summer. **D.** Disc impregnated with thick pigmented extract.

FIG. 3. Zones of inhibition produced by known amounts of solids in decolorized ethyl ether extracts prepared from dried sponge. Material collected in September.